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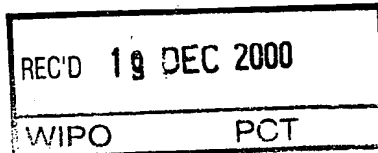
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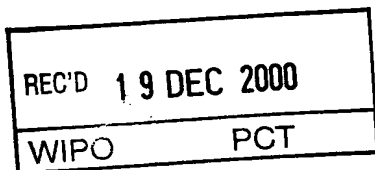
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99116881.6



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Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Applicant(s):
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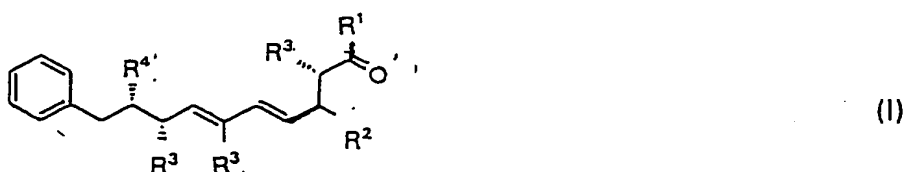
Applicant: Prof. Dr. Daniel R. Dietrich et al.
"Congener independent detection of microcystin and nodularin congeners"
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EPO - Munich
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06. Sep. 1999

Description

The present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I)



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which is part of a group of toxins derived from various cyanobacteria, to a method for its production, to a diagnostic kit containing the proteinaceous compound as well as to a method for substantially decreasing the amount of a compound containing the group represented by formula (I) in fluids.

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Due to increasing settlement, industrialisation and intensive agriculture wide spread problems of water pollution have arisen. This water pollution and the following eutrophication has led in many cases to the development of blooms of blue-green algae (i.e. cyanobacteria). The environmental factors which are

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responsible for the development of such blooms of cyanobacteria are up to now almost unknown. In general, blooms of cyanobacteria can be found in eutrophic bodies of water, e.g. under such conditions as relatively high nutrient levels (phosphate and nitrate), water temperatures of between 15 to 30°C and pH-values of between 6 and 9 or higher (Wicks et al., 1990).

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A severe problem of the development of blooms of cyanobacteria is that cyanobacteria produce a broad variety of toxic substances. Accordingly, since the end of the last century there has been an increasing number of cases of intoxication and even deaths of humans, animals, especially birds and fishes, which could be

demonstrated to be caused by the use of water which was contaminated with cyanobacteria after chlorination and filtration for medical purposes (cases of deaths in the dialysis centers of Caruaru, Brazil, 1996 and Evora, Portugal, 1995), by the consumption of contaminated drinking water or even of clumps of cyanobacteria themselves (Francis, 1878; Falconer et al., 1983; Carmichael, 1984; Beasley et al., 1989; Mahmood et al., 1988; Skolberg et al. 1984).

The toxin producing cyanobacteria can be subdivided into species which synthesize mostly hepatotoxic peptides such as *Microcystis* sp., *Nodularia* sp. and *Oszillatoria* sp., and other genes which produce mostly neurotoxic alkaloids such as *Anabaena* and *Aphanizomenon* (Carmichael et al., 1990). Studies of different strains of *M.aeruginosa* revealed that, depending on strain and habitat, the cyanobacteria produce different congeners and amounts of a toxin (Sivonen et al., 1992 a-c).

Cyanobacteria can secrete the intracellularly produced toxins into the surrounding water (Watanabe et al., 1992 a,b). Further studies showed that the microcystin congener microcystin-LR is photostable, however, it can be microbially degraded (Watanabe et al., 1992 a; Tsuji et al., 1994; Cousins et al., 1996). Under aerobic conditions and in culture media which were inoculated with bacteria, the half-lifetimes of microcystin-LR and -YR were more than 45 days (Watanabe et al., 1992 a). In contrast, half-lifetimes of less than 5 days were determined in seawater (Cousins et al., 1996). Under unfavorable conditions (i.e. cold temperatures and minimal presence of specific populations of microbes) microcystins may persist several days to even months and, therefore, may represent a potential danger for humans via the drinking water supply.

Accordingly, the increased incidence of gastroenteritis and liver carcinomas in humans has been attributed to the consumption of drinking water which was contaminated with cyanobacterial hepatoxins (in particular microcystin-LR) in several studies, although a direct relation between chronic microcystin-LR exposition and the development of liver carcinomas has not yet been proven (Tisdale, 1931; Keleti et al., 1981; Billings, 1981). Clinical indications of micro-

cystin toxicoses in mammals is characterized by weakness, anorexia, mucous pallor, muscle tremor, forced expirations and death by hypovolemic shock which is caused by intrahepatic hemorrhagia and/or liver failure (Theiss et al., 1988; Jackson et al., 1984).

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Mammals seem to take up microcystin orally, and the toxin reaches the liver with the blood via a highly specific transporter mechanism (organic anion carrier) (Eriksson et al, 1990; Hooser et al., 1990; Runnegar et al., 1991). One molecular mechanism of the serious effects of microcystin seems to be its binding to the catalytic subunit of proteinphosphatases 1 and 2A which leads to their inhibition (Eriksson et al., 1990; Yoshezawa et al., 1990; Matsushima et al., 1990; Honkanen et al., 1990; McKintosh et al., 1990; McKintosh et al., 1995; Runnegar et al., 1996). After acute intoxication of high microcystin concentrations, the inhibition of proteinphosphatases leads to hyperphosphorylation of intermediate filaments which, in turn, is followed by collapse of the cytoskeleton, loss of the cells' structure, extensive intrahepatic hemorrhagia and necrosis of the hepatocytes (Eriksson et al., 1990; Falconer et al., 1981, 1992). Similar to other proteinphosphatase inhibitors (e.g. calyculin-A, okadaic acid), the chronic exposition of mice to microcystin-LR leads to promotion of liver tumors (Falconer, 1991; Nishiwaki-Matsushima et al., 1992).

Due to the high toxicity and cancerogenity of hepatotoxic cyanobacteria toxins and the potential chronic exposition of organisms (humans as well as animals) to these toxins via the drinking water there is an urgent need to detect toxic blooms of cyanobacteria early and to decrease the concentration of cyanobacteria toxins in drinking water.

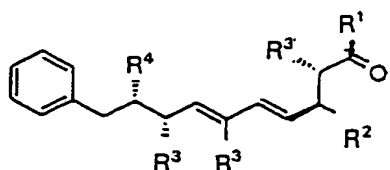
Since it has been difficult to analytically and routinely detect the different microcystin and nodularin congeners with the required sensitivity (Kenefick et al., 1993; Lawton et al., 1994), prior art studies have concentrated on the destruction of the cyanobacteria toxins during the drinking water process. Mostly, continuous methods have been studied which can be carried out under routine conditions such as sand filtration, binding to activated carbon and

destruction by chlorination (James et al., 1994). However, these studies revealed that neither sand filtration nor chlorination, UV-irradiation, treatment with hydrogen peroxide or potassium permanganate nor filtration via activated carbon could substantially remove the cyanobacteria toxins from drinking water. In this case a further problem seems to be the treatment of the raw water which is contaminated with cyanobacteria. The chlorination or the treatment of the cyanobacteria with copper sulfate leads to the release of the cyanobacteria toxins which are present in the cytosol without destroying the toxins to even the lowest degree. Also, the chlorination of sand filtered water is ineffective. Only the filtration via activated carbon seems to be appropriate to remove a considerable amount (about 60% to 80%) of the toxins. However, this purification performance was only reached for a limited period of time due to a relatively quick saturation of the activated carbon particles. Therefore, after about 10,000 bed volumes (1 bed volume = volume of the activated carbon) the filters became leaky.

Therefore, the technical problem underlying the present invention is to provide a novel system for the reliable detection as well as the removal of all kinds of hepatotoxic cyanobacteria toxins such as microcystin and nodularin congeners, particularly in and from, drinking water and other sources.

The solution to the above problem is provided by the embodiments of the present invention as characterized in the claims.

In particular, the present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I).



(I)

which is part of a toxin derived from a cyanobacterium, wherein group R¹ represents -OR' or -NR'₂ and group R² represents hydrogen, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, (C₁-C₄)acyl, (C₁-C₄)aminoacyl or (C₁-C₄)carboxaminoacyl, or the groups R¹ and R² are connected to each other to form a cyclic compound, the groups R³ which may be the same or different are each independently selected from the group consisting of hydrogen and (C₁-C₄)alkyl, group R⁴ represents (C₁-C₄)alkoxy, and wherein the phenyl group may be substituted or unsubstituted.

The term "proteinaceous compound or functionally active derivate or part thereof" means a compound which is capable of binding the above-described group of formula (I) and substantially consists of one or more polypeptides. The functionally active form of the proteinaceous compound according to the present invention may be a monomeric or a homo- or heterodimeric, -trimeric, -tetrameric or other oligomeric form.

The term "binding site" for the group as defined above means a three-dimensional arrangement of atoms of the above proteinaceous compound which is able to specifically interact with the group of formula (I) as defined above. The specific interaction may be any kind of chemical and/or physical interaction and comprises covalent binding, electrostatic interactions, hydrogen bonding, Van-der-Waals- as well as hydrophobic interactions.

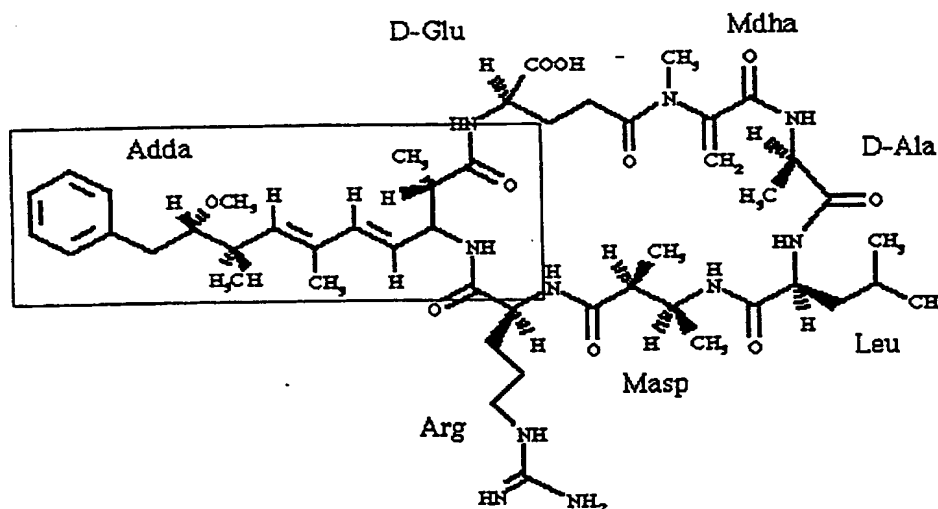
Preferably, the group R' in the formula (I) represents independently from each other hydrogen, substituted or unsubstituted (C₁-C₄)alkyl or (C₁-C₄)acyl, when bound to nitrogen. According to a further preferred embodiment of the proteinaceous compound as defined above, the groups R³ in the above formula (I) each represent methyl and group R⁴ represents methoxy.

According to a further preferred embodiment of the proteinaceous compound of the present invention, group R¹ represents aminoacyl and group R² represents (C₁-C₄)acyl, or group R¹ represents glycyl or D-alanyl, respectively, and group R²

represents acetyl, or group R^1 represents $-NH_2$ and group R^2 represents glutamyl or 2-aminopropionamidyl, respectively.

Preferably, the group represented by the above formula (I) is part of a toxin selected from the group consisting of microcystin and nodularin congeners. Microcystin (MC) congeners are hereinafter referred to as microcystin-XY.

The chemical structures of *M. aeruginosa* and *Nodularia* sp.-hepatotoxins (i.e. microcystin-XY and nodularin) are described in several prior art studies (Botes et al., 1982 a, d, 1994, 1985; Rinehard et al., 1988). Microcystin-XY and nodularin are cyclic peptides consisting of seven or five, respectively, amino acids. The following formula represents microcystin-LR.



Nodularin and microcystin-XY share the same specific characteristic amino acid (ADDA). The basic structure of microcystin-XY congeners consists of five non-variable amino acids: β -methylasparaginic acid, alanine, N-methyl-dehydroalanine, glutamate, and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienic acid (ADDA). The differences between individual microcystin congeners are based on the two variable L-amino acids which are, for example, L-arginine and

L-leucine in microcystin-LR and two times L-arginine in microcystin-RR, respectively. Normally, cyanobacteria produce a mixture of different forms of the toxins. The isolation of microcystin-XY from natural blooms of blue-green-algae resulted in up to six different microcystin congeners, and toxin concentrations up to 10 mg per g of dry mass of algae were determined (Wicks et al., 1990; Tsuji et al., 1994; Tencallar et al., 1994, 1995).

An especially preferred example of the proteinaceous compound according to the present invention is a polyclonal or monoclonal antibody which is directed to the group represented by the above formula (I).

The proteinaceous compound according to the present invention, e.g. a polyclonal or monoclonal antibody, has the advantage to be capable of binding to all congeners of the cyanobacteria hepatotoxins, e.g. microcystin and nodularin congeners.

In contrast to the proteinaceous compound of the present invention, the commercially available antibodies or ELISA kits, respectively, are only capable of recognizing a very limited number of microcystin congeners (up to 2). This means that the toxicity of blooms of cyanobacteria can be massively underdetermined by the use of the antibodies or kits, respectively, known so far.

A further embodiment of the present invention relates to a method for the preparation of the proteinaceous compound as defined above, comprising the steps of

- a) preparing a compound containing a group represented by the formula (I) as defined above and
- (b) coupling the compound of step (a) to a carrier.

Carriers which are suitable for the above method may be selected from the group consisting of polyethyleneglycol, proteins and polysaccharids. Preferably, the protein carrier is selected from bovine serum albumin (BSA), ovalbumin (OVA) and chloramphenicol acetyl transferase (Cat).

In another preferred embodiment of the present invention, the above method further comprises the steps of

- (c) immunizing an animal with the conjugate obtained in step (b) above and
- 5 (d) isolating the animal's blood serum.

A further embodiment of the present invention relates to a diagnostic kit containing the proteinaceous compound as defined above. The proteinaceous compound according to the present invention, e.g. a polyclonal or monoclonal
10 antibody, is particularly useful for the detection of a compound containing the group represented by the above formula (I) as well as to substantially decrease the amount of a compound containing the group represented by the formula (I) in a fluid.

15 Therefore, a further embodiment of the present invention relates to a method for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid, e.g. water such as hemodialysis water, drinking water or water derived from rivers, lakes and oceans, comprising the steps of

- 20 (a) preparing the proteinaceous compound as defined above,
- (b) coupling the compound obtained in step (a) to a polymeric matrix, and
- (c) contacting the fluid with the polymeric matrix obtained in step (b).

25 Furthermore, the above method may also be applied to the cleaning of any other sources of cyanobacteria toxins such as, for example, food stuffs.

The Figures show:

30 Fig. 1 is a diagramm showing a flow chart for the strategy of preparation of an anti-ADDA antibody according to the present invention.

Fig. 2 is a diagramm showing preferred strategies for the coupling of an ADDA-hapten to a protein.

Fig. 3 shows several ADDA-derivatives which were synthesized for the production of the antibody useful for congener independent detection of microcystin and nodularin congeners.

Fig. 4 is a diagramm showing the general principle of the indirect competitive microcystin enzyme-linked immunosorbent assay (MC-ELISA).

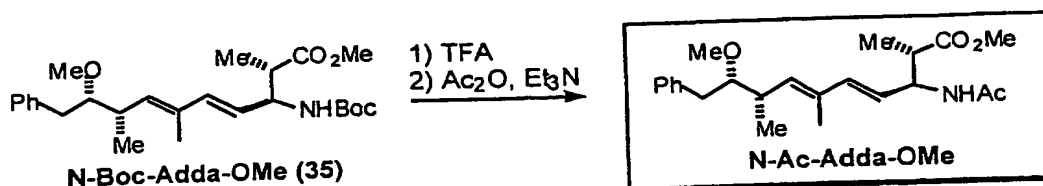
Fig. 5 is a diagramm showing the crossreactivity with respect to different microcystin congeners (MC-LR, -RR and -YR) and nodularin of the anti-ADDA antibody (ADDA-824new) raised in sheep which is directed against ADDA-HG coupled to ovalbumin.

The present invention is further illustrated by the following non-limiting example.

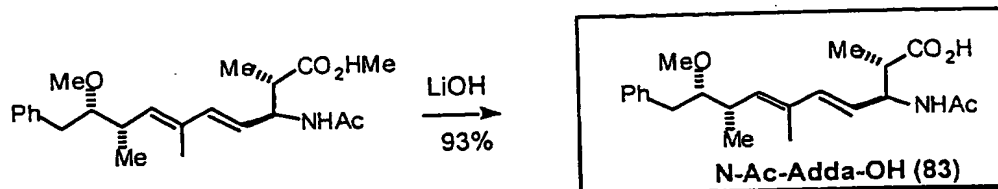
EXAMPLE

ADDA hapten synthesis

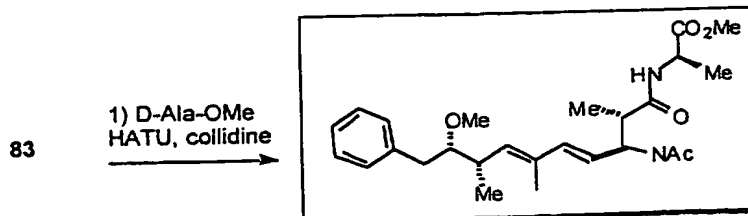
The starting material N-Boc-ADDA-Me (35) was prepared by the published route: Humphrey, J. M.; Aggen, J.; Chamberlin, A. R. *J. Am Chem. Soc.* 1996, 118, 11759-11770. "Synthesis of the Serine-threonine Phosphatase Inhibitor Microcystin LA."



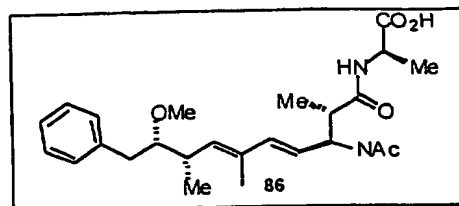
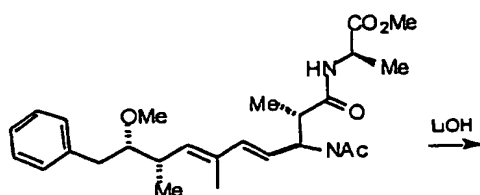
N-Ac-ADDA-OMe. To 31 mg (0.70 mmol) of **Boc-ADDA-OMe** in a flask was added 2 mL of TFA. After one hour the TFA was removed under vacuum, and the residue was concentrated three times from toluene to remove the TFA. The resulting oil was dissolved in 2.5 mL of freshly distilled CH_2Cl_2 , and this was cooled to 0° C. 28 mg (0.28 mmol) of anhydrous triethylamine was added to the solution, followed by 0.141 g (1.39 mmol) of freshly distilled acetic anhydride. One hour later 5 mL of saturated NH_4Cl was added, and the mixture was stirred for 20 minutes at 0° C. The mixture was partitioned between water and EtOAc, and the phases were separated. The aqueous phase was extracted twice with EtOAc. The combined organic phases were washed once each with 50% saturated NH_4Cl , 50% saturated NaHCO_3 , and brine, dried over MgSO_4 , filtered, and concentrated under vacuum to give a white solid. The solid was purified via flash chromatography (1/1 EtOAc/hexanes) to give 25 mg (93%) of a white solid: R_f 0.23 (40:60 EtOAc:hexanes); IR (thin film) 3330, 2919, 1731, 1654, 1454 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 0.99 (d, J = 6.5, 3H), 1.20 (d, J = 7, 3H), 1.57 (s, 3H), 2.02 (s, 3H), 2.58 (ddq, J = 6, 6.5, 10 Hz, 1H), 2.67 (dd J = 7.5, 14 Hz, 1H), 2.78 (obscured mult., 3H), 2.79 (dd, J = 5, 13.5 Hz, 1H), 3.17 (ddd, J = 5, 6, 7 Hz, 1H), 3.21 (s, 3H), 3.65 (s, 3H), 4.71 (ddd, J = 4.5, 5.5 Hz, 1H), 5.37 (d, J = 9.5 Hz, 1H), 5.42 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.40 (d, J = 9 Hz, 1H), 7.25-7.15 (m, 5H); ^{13}C NMR (125 MHz, CDCl_3) δ 175.8, 169.5, 139.5, 136.6, 136.3, 132.4, 129.5, 128.2, 126.0, 124.9, 87.1, 58.6, 3.0, 51.7, 43.6, 38.4, 36.8, 23.5, 16.2, 14.9, 12.7; HRMS calcd for $\text{C}_{23}\text{H}_{34}\text{NO}_4$ ($\text{M} + \text{H}$) $^+$: 388.2488. Found: 388.2505.



N-Ac-ADDA-OH. To 22 mg (0.057 mmol) of the protected ADDA-derivative in 2 mL THF was added 0.57 mL (0.57 mmol) of 1 M LiOH. After 22 hours the mixture had clarified, and it was partitioned between hexanes and water. The phases were separated, and the aqueous phase was washed once with hexanes. The combined organic phases were back-extracted three times with water. The combined aqueous phases were acidified with 1 M NaHSO₄, and extracted three times with CH₂Cl₂. The combined CH₂Cl₂ phases were washed once with brine, filtered through cotton, and concentrated to give 23 mg of **83** as an oil that was taken on without purification: R_f 0.34 (1:49:50 HO-Ac:EtOAc:hexanes); IR (thin film) 3295 br, 2923, 1713, 1640 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 0.99 (d, J = 6.5, 3H), 1.25 (d, J = 7, 3H), 1.58 (s, 3H), 2.02 (s, 3H), 2.57 (ddq, J = 6.5, 6.5, 9.5 Hz, 1H), 2.65 (dd J = 7.5, 14 Hz, 1H), 2.76 (par.obsc. m, 3H), 2.77 (dd, J = 5, 13 Hz, 1H), 3.17 (ddd, J = 5, 6.5, 6.5 Hz, 1H), 3.21 (s, 3H), 4.71 (ddd, J = 5, 6, 10 Hz, 1H), 5.37 (d, J = 9.5 Hz, 1H), 5.45 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.37 (d, J = 9.5 Hz, 1H), 7.25-7.15 (m, 5H); HRMS calcd for C₂₂H₃₂NO₄ (M+H)⁺: 374.2331, Found: 374.2325.



N-Ac-ADDA-D-Ala-OMe. To 17 mg (0.12 mmol) of D-Ala-OMe hydrochloride and 14 mg (0.036 mmol) of HATU in a flask was added 9 mg (0.024 mmol) of **83** in 0.6 mL DMF. The resulting solution was cooled to 0° C, and 41 mg (0.34 mmol) of collidine was added. The solution was stirred at 0° C for 2 hours, followed by warming to room temperature and stirring overnight. The mixture was partitioned between water and EtOAc, and the phases were separated. The aqueous phase was extracted once with EtOAc. The combined organic phases were washed once each with sat. NaHCO₃, water, 1 M NaHSO₄, water, and brine, dried over MgSO₄, filtered, and concentrated under vacuum to an off-white solid. Chromatography (80:20 EtOAc:hexanes) gave 8 mg (73%) of a white solid: R_f 0.17 (60:40 EtOAc:hexanes); IR (thin film) 3284, 3067, 2923, 1743, 1650, 1542 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 0.99 (d, J = 6.5, 3H), 1.23 (d, J = 7, 3H), 1.35 (d, J = 7 Hz, 3H), 1.58 (s, 3H), 2.04 (s, 3H), 2.52 (dq, J = 4, 7 Hz, 1H), 2.59 (ddq, J = 6.5, 7, 9.5 Hz, 1H), 2.68 (dd J = 7.5, 14 Hz, 1H), 2.81 (dd, J = 4.5, 14 Hz, 1H), 3.19 (ddd, J = 5, 7, 7 Hz, 1H), 3.22 (s, 3H), 3.75 (s, 3H), 4.55 (dq, J = 7, 7 Hz, 1H), 4.62 (m, 1H), 5.39 (d, J = 9.5 Hz, 1H), 5.45 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.23 (d, J = 7 Hz, 1H), 7.05 (d, J = 9 Hz, 1H), 7.27-7.17 (m, 5H); ¹³C NMR (125 MHz, CDCl₃) δ 12.7, 15.4, 16.2, 18.4, 23.5, 36.7, 38.2, 44.4, 47.9, 52.6, 53.7, 58.6, 86.9, 125.2, 125.9, 128.2, 129.4, 132.2, 136.2, 139.4, 169.9, 173.2, 174.6; HRMS calcd for C₂₆H₃₉N₂O₅ (M+H)⁺: 459.2859, Found: 459.2869.



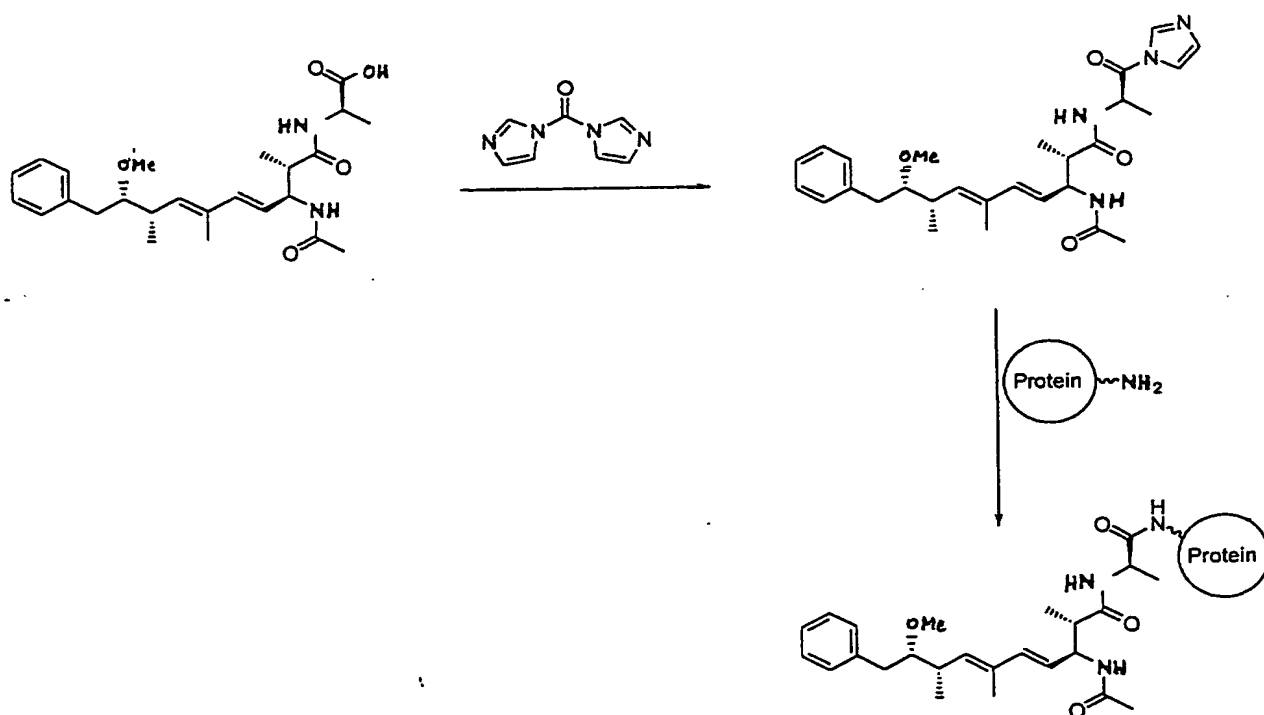
N-Ac-ADDA-D-Ala-OH. To 5 mg (0.011 mmol) of N-Ac-ADDA-D-Ala-OMe in 1.75 mL of THF was added 0.10 mL (0.10 mmol) of 1 M LiOH. After 50 minutes, the mixture was partitioned between ether and water, and the phases were separated. The aqueous phase was washed once with ether. The combined ethereal phases were back-extracted three times with water, and the combined aqueous phases were acidified to pH = 3 with saturated citric acid. The aqueous phases were then extracted twice with EtOAc, and the combined EtOAc phases were washed twice with water, once with brine, dried over MgSO₄, filtered, and concentrated *in vacuo*. The resulting solid was purified by preparative reversed-phase HPLC, retention time of product = 15.7 minutes (70 MeOH / 30 0.2% aq. TFA), to give 4 mg (85%) of the title compound as a white solid: R_f 0.36 (1 HOAc / 10 MeOH / 89 CH₂Cl₂); IR (thin film) 3288 br, 2937, 1720, 1658, 1632 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.94 (d, *J* = 7.0 Hz, 3H), 0.96 (d, *J* = 7.0 Hz, 3H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.52 (s, 3H), 1.82 (s, 3H), 2.63 (dd, *J* = 7.0, 14.0 Hz, 1H), 2.73 (dd, *J* = 5.0, 14.0 Hz, 1H), 3.16 (s, 3H), 3.22 (ddd, *J* = 5.5, 5.5, 6.5 Hz, 1H), 4.19 (dq, *J* = 7.0, 7.5 Hz, 1H), 4.40 (m, 1H), 5.38 (d, *J* = 10.0 Hz, 1H), 5.44 (dd, *J* = 6.5, 16.0 Hz, 1H), 6.05 (d, *J* = 16.0 Hz, 1H), 7.17 (d, *J* = 7.5 Hz, 3H), 7.25 (t, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 9.0 Hz, 1H), 8.01 (d, *J* = 7.0 Hz, 1H); FAB MS calcd for C₂₅H₃₇N₂O₅ (M + H)⁺: 445.2702. Found: 445.2695.

Coupling of hapten to proteins

Preparation of BSA-, cBSA-, and OVA-N-AcADDA-AlaOH.

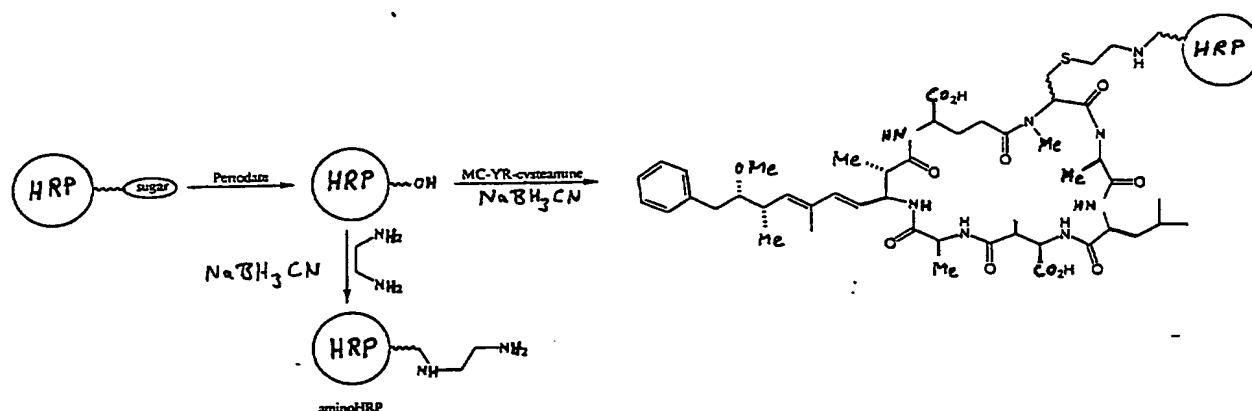
BSA (10.6 mg), cationised BSA (cBSA) (10.0 mg), and OVA (8.3 mg) were each dissolved in PBS (1000 μL). Carbonyldiimidazole (19.81 mg, 0.12 mmol) was

dissolved in dry DMF (500 μ L), and a portion of the solution (100 μ L) was added to *N*-acetyl-ADDA-D-Ala-OH (1.0 mg, 2.2 μ mol) and allowed to stand for 90 min. DMF was added (BSA, 260 μ L; cBSA, 260 μ L; OVA, 280 μ L) to the protein solutions just prior to addition of the activated ADDA-derivative. The solution of the activated ADDA-derivative (40 μ L each to the BSA and cBSA, 20 μ L to the OVA) was then added to the protein solutions, and the reaction was allowed to proceed at 4 °C for ca 16 h. The resulting conjugates were repeatedly diluted and then concentrated by ultrafiltration (Filtron centrifugal ultrafiltration tubes, 10K cutoff) until the calculated dilution of unretained low molecular weight compounds was $> 10^6$.



Preparation of HRP-MC-YR and aminoHRP.

Horse radish peroxidase (HRP) was oxidized by the method of Hermanson. HRP (19.73 mg, Boehringer) was dissolved in PBS and cooled to 4 °C. NaIO₄ (36.7 mg) was dissolved in water (2 mL), and 100 µL of this was added to the HRP solution, which rapidly became green. The reaction was held at 4 °C in the dark for 20 min, then the HRP was separated from low molecular weight material by elution with PBS through a desalting column (Bio-Rad Econo-Pac 10DG).

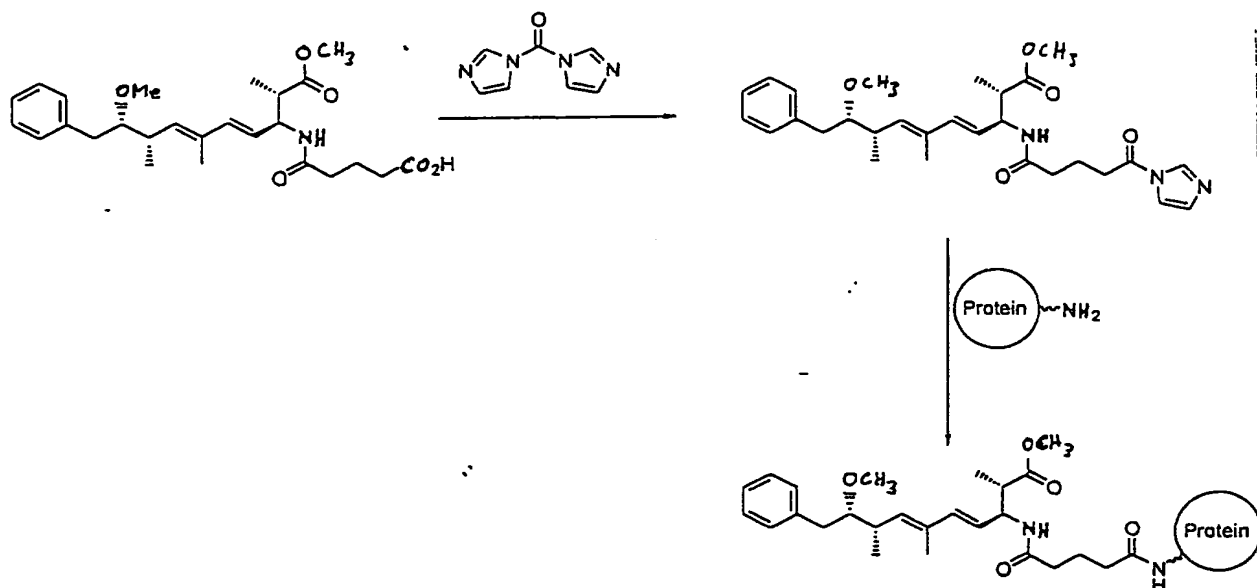


To half of the oxidized HRP MC-YR-cysteamine (51 µg, see below) was added in MeOH (50 µL). To the other half diaminoethane hydrochloride (500 mg) was added in PBS (500 µL). NaBH₃CN (16.4 mg) was dissolved in PBS (500 µL), and 100 µL of this was added to each HRP reaction (which immediately became crimson). After standing at 4 °C in the dark for about 16 h, the reactions were quenched by addition of diethanolamine in PBS (50 µL of 300 µL of diethanolamine in 5 mL PBS) and allowed to stand at 4 °C in the dark for 2 h. The HRP solutions were then purified by passing through desalting columns (as above). The diaminethane conjugate (henceforth referred to as aminoHRP) and MC-YR conjugates were further purified by ultrafiltration to > 10⁴ dilution (as above).

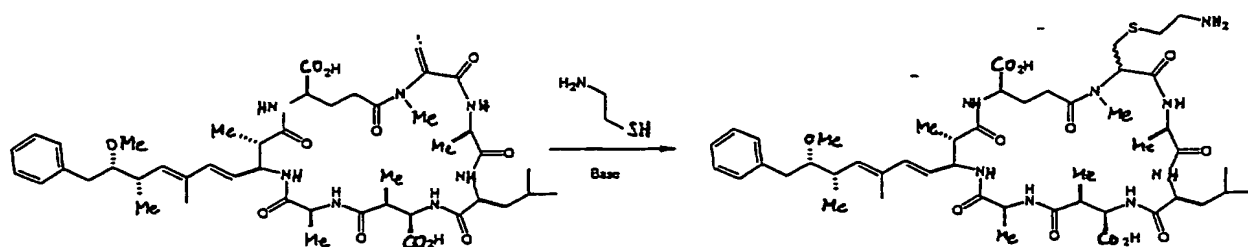
Preparation of HRP-, aminoHRP-, and OVA-ADDA-HG.

HRP, aminoHRP, and OVA were each dissolved in PBS (1 mL). To Me-ADDA-HG (0.67 mg) was added CDI (1.16 mg) in dry DMF (100 µL), and the reaction proceeded at ambient temperature 1.5 h whereupon dry DMF (150 µL) was

added. A portion of this solution was added to the solutions of the proteins (50 μ L to aminoHRP, 100 μ L to HRP and OVA). DMSO (200 μ L) was then added to the HRP and OVA reactions to assist in solubilising the reactants, and the three reactions were maintained at 4 °C in the dark for ca 16 h. The conjugates were then purified on the desalting column and then further purified by repeated ultracentrifugation to $> 3 \times 10^4$ dilution (as above).



Preparation of MC-YR-cysteamine conjugate.



The method is based on those of Kondo et al. (1992) and Sherlock et al. (1998). Cysteamine (15.6 mg) was dissolved in water (500 μ L), and MC-YR (500 μ g) was dissolved in 5% K₂CO₃ (500 μ L). The cysteamine solution (50 μ L, followed

by 100 μ L at 30 min) was added to the MC-YR solution in portions. After about 2 h the reaction was acidified to pH 3 to 4 and applied to a reverse-phase flash column (4 \times 1 cm). The column was eluted successively with water (10 mL), 10% MeOH (10 mL), 20% MeOH (10 mL), 30% MeOH (10 mL), 50% MeOH (2 \times 10 mL), 70% MeOH (2 \times 10 mL), and MeOH (3 \times 10 mL). HPLC analysis indicated the product to be in the 50% MeOH and the first of the 70% MeOH fractions. These fractions were combined and the solvent removed *in vacuo* to yield MC-YR-cysteamine as a colourless solid (204 μ g). ESI-MS *m/z* 1121.9 (M-H⁺); ¹H, COSY and HMBC NMR spectra were consistent with the desired product.

Immunization of sheep and mice with ADDA-protein conjugates

Nine sheeps and nine mice were immunized with of the above BSA-ADDA-, Cat-ADDA- and OVA-ADDA-conjugates, respectively (three animals for each conjugate). 1 mg of each conjugate was used in a volume of 1 ml and Freund's complete adjuvans was added to a final of 2 ml in case of the primary injection, and incomplete adjuvans was added to the same volume in case of boost injections (3 boosts in case of sheeps, 6 boosts in case of mice).

ELISA

ELISA plates (NUNC MaxiSorp 1 #439454, Denmark) were coated with OVA-N-acetyl-D-alanyl-ADDA conjugate in 0.05 M sodium bicarbonate buffer pH 9.6 (50 μ l, 2.5 μ g/ml) overnight at 20°C. After a wash with PBS, additional binding sites were blocked by incubation with OVA (1% w/v, 300 μ l, 1 h, 20–25 °C). Plates were washed three times with PBS and used immediately or stored at 4 °C for up to 7 days. In the assay, sample or standard (50 μ l) was added to the wells together with antiserum at the appropriate dilution (50 μ l, e.g. 1/75000 according to Fig. 5). After incubation at 20–25 °C for 2 h, wells were washed twice with PBS + 0.05% Tween™ 20 (PBST) and twice with PBS. Anti-sheep secondary antibody (100 μ l, 1/10000) was then added to the wells and incubated for 2 h. Thereafter, wells were aspirated, washed twice with PBST and

twice with PBS. TMB substrate solution, prepared by adding 110 μ l TMB stock (10 mg/ml in DMSO) to 11 ml of sodium acetate buffer (0.1 M, pH 5.5) containing 0.005% H_2O_2 , was then added and incubated for 15 min. The reaction was stopped by addition of H_2SO_4 (50 μ l, 2 M), and the absorbance was determined with a microplate spectrophotometer set at 450 nm. Standards and samples were prepared for ELISA by dilution in the following diluents: (i) methanol in PBS to a maximum methanol concentration of 10% (v/v); (ii) PBS; (iii) lake water (lake constance, Germany); (iv) tap-water. All samples were analysed in at least duplicate, and over a range of dilutions.

ELISA method in detail

The principle of the indirect competitive microcystin ELISA (MC-ELISA) employed is shown in Fig. 4. This method comprises the following steps:

- 1) Prepare antigen (ADDA-BSA) in bicarbonate buffer, pH 9.4;
at 2.5 μ g/ml (5ml + /plate)
- 1) Coat antigen onto microtitre plate at 50 μ l/well, tap to mix and cover,
incubate overnight at room temperature (RT, 22°C).
- 2) Wash 2 times in PBS.
- 3) Block plate with 1% OVA - (300 μ l for 1 hour at RT (22°C)).
- 4) Wash 2 times in PBS, aspirate and use or add 2-300 μ l PBS for storage.

The plates can be stored at this stage in PBS (at 4°C) for up to 7 days.

- 5) Add 50 μ l sample, or standard, in PBS;

and 50 μ l of antibody **ADDA-#824** (developed in sheep) at 1/75000 dilution in OVA-blocker and incubate for 2 hours at RT (22°C).

Standard curve: Primary 5000ng/ml, then 9 serial 1:7,5 dilutions (1/4) in PBS;

6) Wash 2 times in PBS/Tween, 2 times in PBS.

5 7) Add 100 μ l of secondary antibody conjugate diluted in OVA (peroxidase-conjugated rabbit-anti-sheep IgG (from ICN#55814) at a final dilution of 1/10000) and incubate for 2 hours at RT (22°C).

8) Wash 2 times in PBS/Tween, 2 times in PBS.

10

9) Turn on plate reader - needs a 15min warm up before reading at step 12.

10) Add 100 μ l of substrate. Incubate at RT (22°C) until colour develops (15 min).

15

11) Add 50 μ l stop solution (2M H₂SO₄)

12) Read Absorbance at 450nm. Note that the absorbance at 655nm can be measured prior to adding the stop solution if required.

20

Preparation of buffers:

Bicarbonate coating buffer

Dissolve 0.85g Na₂CO₃, (or 2.15g Na₂CO₃.2H₂O) and 1.47g NaHCO₃ in 500ml distilled water, adjust pH to 9.6 (gives 0.05M bicarbonate).

25

Phosphate Buffered Saline (PBS)

To prepare 10 times stock solution:

NaH₂PO₄.2H₂O 2.897g (or NaH₂PO₄ anhydrous 2.06g)

Na₂HPO₄ anhydrous 11.938g

30

NaCl 87.660g

Weigh phosphates, add water to 800ml, adjust pH to 7.4, then add salt.

Add water to 1l and check pH (must be 7.2 to 7.6).

Dilute 1/10 for use: gives 0.01M wrt phosphate and 0.15M NaCl.

Ref.: Mishell et al. (1980)

5 *PBS-Tween*

Suspend Tween-20 at 0.05% in PBS (0.5ml/l);

Use for the washing steps 6) and 8), respectively, described above.

OVA-blocking buffer

10 Dissolve OVA (Sigma A-5503) in PBS at 1% (2g/200ml).

Use for blocking plates, and as diluent for Ab and Ab".

Secondary antibody

15 Also referred to herein as Ab", HRP-conjugate, and Second Ab. The dilution depends on the batch used, but approximate dilutions are as follows:

ICN HRP-conjugated Rabbit-anti-SHEEP-IgG #55814

Use at a working dilution of 1/3000. Stock solution is stored at 1/10 in PBS thiomersal (0.02%).

20

TMB Substrate

Prepare stocks of:

1) Sodium acetate buffer 0.1M, pH 5.5 (1.315g/200ml) (check for precipitate before use).

25 2) TMB (3,3',5,5'-tetramethylbenzidine) at 10mg/ml DMSO; [store in the dark at RT (22°C)].

Immediately before use:

Dissolve 110µl TMB solution (2) in 11ml sodium acetate buffer (1), and, add 165µl H₂O₂ (prepared freshly by diluting 38µl 30% H₂O₂ (commercial strength) into 2.5ml distilled H₂O).

30

ELISA Plates

96-well-plates were from NUNC (Maxisorp I plates, catalogue #439454).

Characterization of polyclonal anti-ADDA-antibody developed in sheep

The optimal concentrations of assay reagents were determined empirically by chequerboard titrations. Assay standard curves were calculated using Microsoft Excel. Cutoff values of 20 to 80% of maximum absorbance were used in order to determine the working range. Cross-reactivity of the assay was determined against congeners of MC-LR, -RR, -YR, -LW, -LF, -LA, desmethyl-MC-LR, desmethyl-MC-RR and nodularin and calculated from the concentration of analogue giving 50% inhibition (I_{50}) of binding to the protein-ADDA solid phase, expressed relative to the I_{50} for free microcystin-LR. The calculation of the cross-reactivity demonstrates that for sample concentrations ranging between 0.01 and 1 ng/ml the actual toxin concentrations are underestimated in the worst case by 10%. As of a sample concentration of 1 ng/ml or 1 μ g/l, all congeners tested are detected with equal sensitivity i.e. 100% cross-reactivity or detection is guaranteed (Fig. 5).

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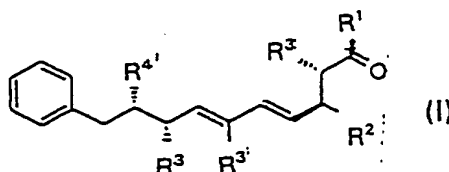
Applicant: Prof. Dr. Daniel R. Dietrich et al.
 "Congener independent detection of microcystin and nodularin congeners"
 Our Ref: D 2681EU - py / js

EPO - Munich
 57

06. Sep. 1999

Claims

1. A proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I)



which is part of a toxin derived from a cyanobacterium, wherein group R¹ represents -OR' or -NR'₂ and group R² represents hydrogen, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, (C₁-C₄)acyl, (C₁-C₄)aminoacyl or (C₁-C₄)carboxyaminoacyl, or the groups R¹ and R² are connected to each other to form a cyclic moiety, the groups R³ which may be the same or different are each independently selected from the group consisting of hydrogen and (C₁-C₄)alkyl, group R⁴ represents (C₁-C₄)alkoxy, - and wherein the phenyl group may be substituted or unsubstituted.

2. The proteinaceous compound of claim 1, wherein the groups R' represent independently from each other hydrogen, substituted or unsubstituted (C₁-C₄)alkyl or (C₁-C₄)acyl, when bound to nitrogen.
3. The proteinaceous compound of claim 1 or 2, wherein the groups R³ each represent methyl and group R⁴ represents methoxy.

4. The proteinaceous compound according to anyone of claims 1 to 3, wherein group R^1 represents aminoacyl and group R^2 represents (C_1-C_4) acyl.
5. The proteinaceous compound of claim 4, wherein group R^1 represents glycyI or D-alanyl and group R^2 represents acetyl.
6. The proteinaceous compound according to anyone of claims 1 to 5, wherein group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl.
7. The proteinaceous compound according to anyone of claims 1 to 6, wherein the toxin is selected from the group consisting of microcystin and nodularin congeners.
8. The proteinaceous compound according to anyone of claims 1 to 7 which is a polyclonal or monoclonal antibody.
9. A method for the preparation of the proteinaceous compound according to anyone of claims 1 to 8 comprising the steps of
- (a) preparing a compound containing a group represented by formula (I) as defined in anyone of claims 1 to 7, and
- (b) coupling the compound of step (a) to a carrier.
10. The method of claim 9, wherein the carrier is selected from the group consisting of polyethyleneglycol, proteins and polysaccharids.
11. The method of claim 10, wherein the protein carrier is selected from bovine serum albumin, ovalbumin and chloramphenicol acetyl transferase.
12. The method according to anyone of claims 9 to 11 which further comprises the steps of
- (c) immunizing an animal with the conjugate obtained in step (b), and
- (d) isolating the animal's blood serum.

13. A diagnostic kit containing the proteinaceous compound according to anyone claims 1 to 8.
- 5 14. Use of the proteinaceous compound according to anyone of claims 1 to 8 for the detection of a compound containing the group represented by the formula (I).
- 10 15. A method for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid comprising the steps of
- (a) preparing the proteinaceous compound according to anyone of claims 1 to 8,
 - (b) coupling the compound obtained in step (a) to a polymeric matrix, and
 - 15 (c) contacting the fluid with the polymeric matrix obtained in step (b).
16. The method of claim 15, wherein the fluid is hemodialysis water, drinking water or water derived from rivers, lakes and oceans.

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Preparation of Anti-ADDA Antibody

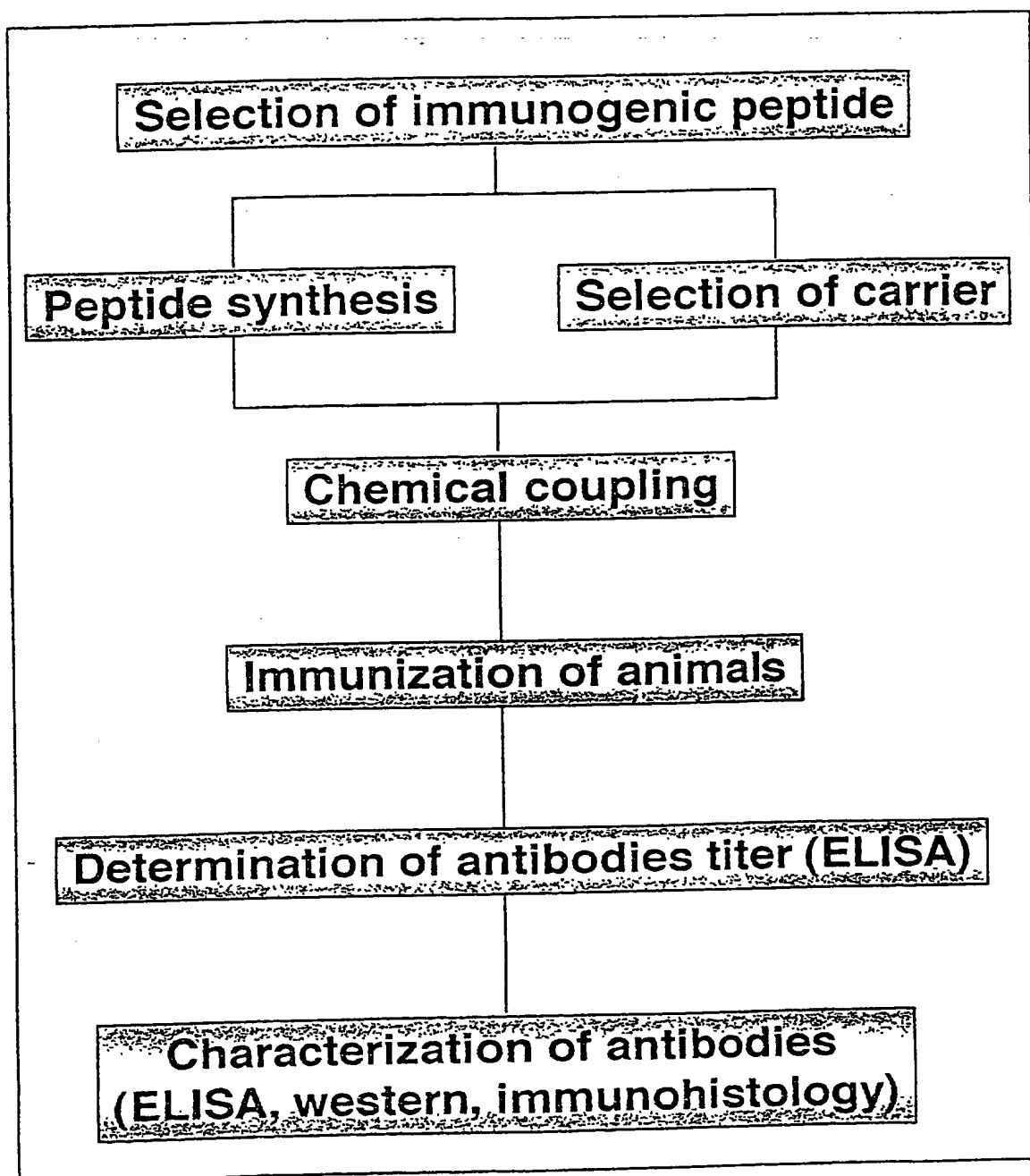
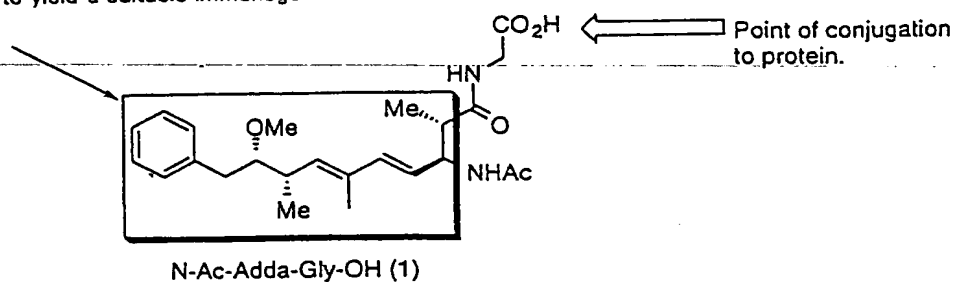


Fig. 1

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Desired are antibodies to this part. Therefore, this part has to be kept constant and the chemistry which is used to couple the ADDA-hapten to the protein has to be varied in order to yield a suitable immunogen and antigen.



Or any other simple amide

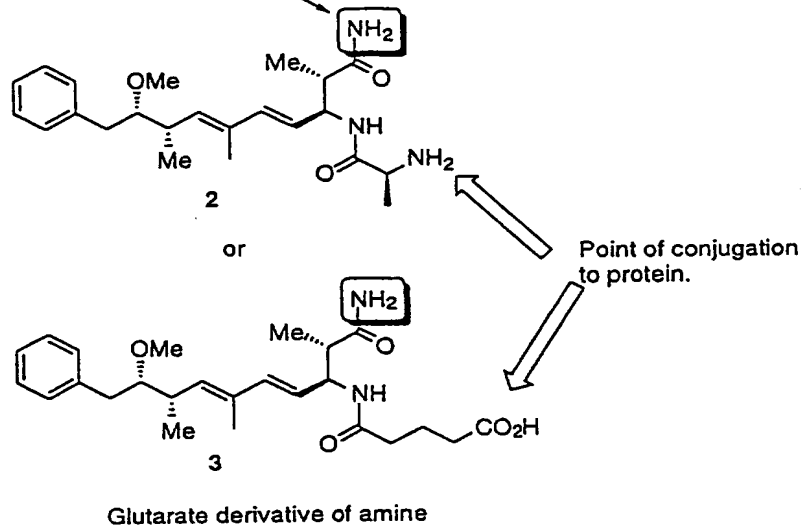
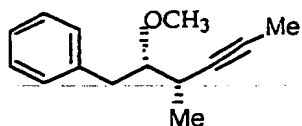


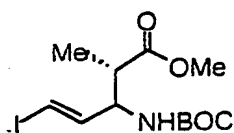
Fig. 2

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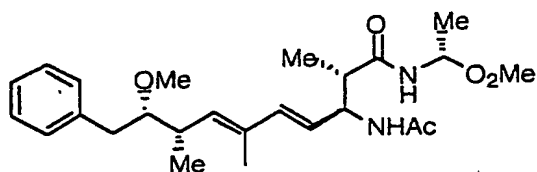
ADDA-Derivatives



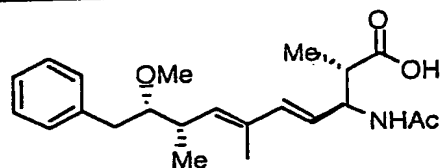
„Alkyne“-precursor
MW 202,32



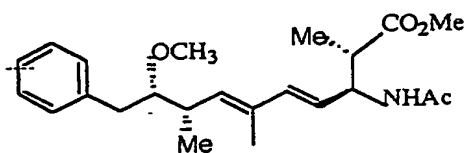
„Vinyl iodide“-precursor
MW 369,23



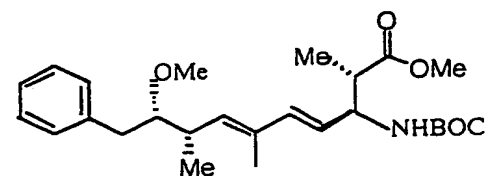
„ADDA“, N-acetyl, D-ala,
methyl ester
MW 458,65



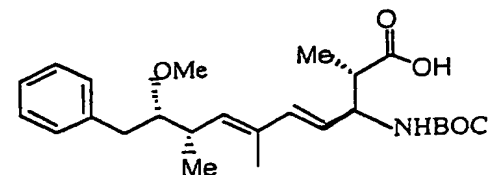
„ADDA“, N-acetyl, free acid
MW 373,54



„ADDA“, N-acetyl, methyl ester
MW 387,57



„ADDA“, BOC-amine, methyl ester
MW 445,66



„ADDA“, BOC-amine, free acid
MW 431,63

Fig. 3

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Indirect Competitive MC-ELISA

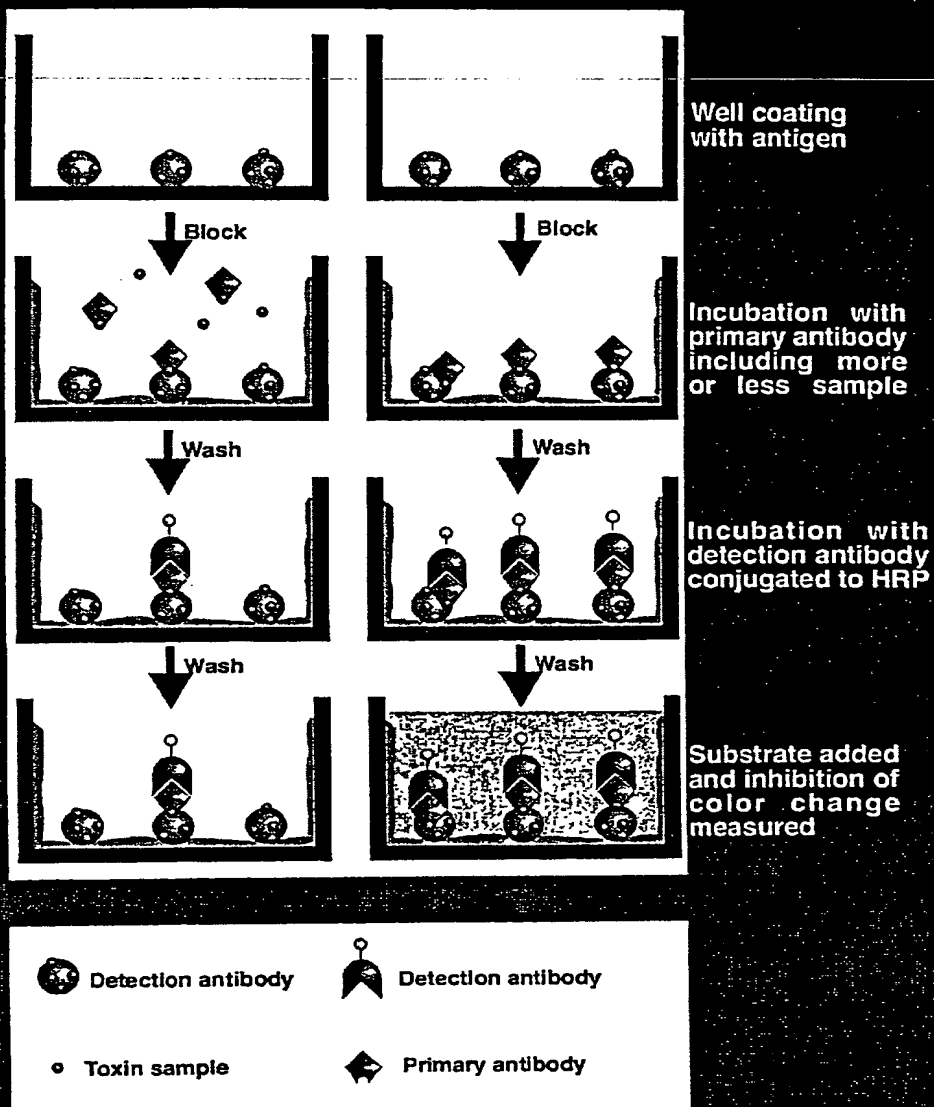


Fig. 4

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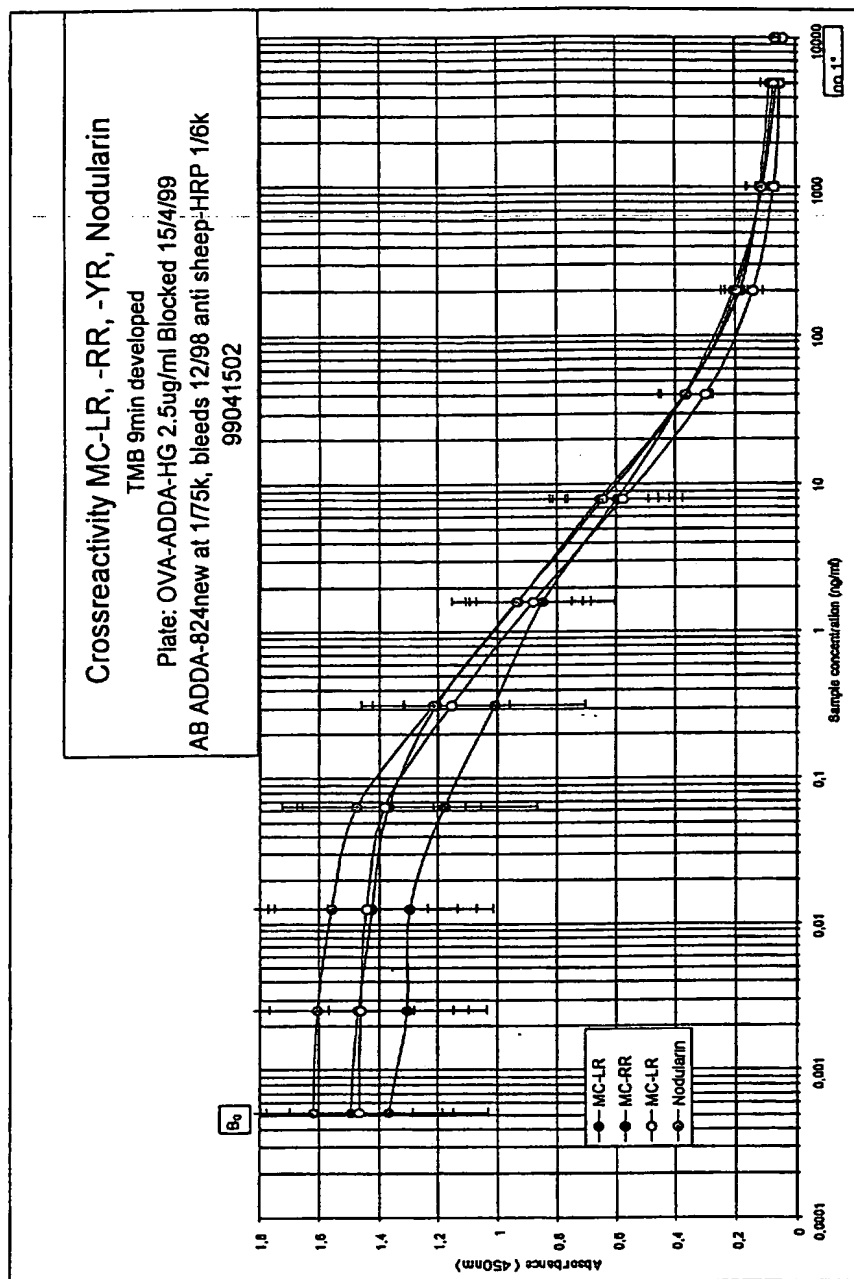


Fig. 5

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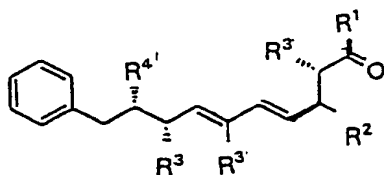
Applicant: Prof. Dr. Daniel R. Dietrich et al.
"Congener independent detection of microcystin and nodularin congeners"
Our Ref: D 2681EU - py / js

Abstract

EPO-Munich
57

06. Sep. 1999

The present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I)



(I)

which is part of a group of toxins derived from various cyanobacteria, to a method for its production, to a diagnostic kit containing the proteinaceous compound as well as to a method for substantially decreasing the amount of a compound containing the group represented by formula (I) in fluids.

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